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TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED OFFICE (DO/US)

PCT/SE99/00749	04 May 1999	15 May 1998 and 22 June 1998
International Application Number	International Filing Date	Priority Date(s) Claimed

NEW ASSAY
Title of Invention

DESOUSA, Sunita and PRAHLAD, Dwarakanath

Applicant(s) for DO/US

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To the United States Designated Office (DO/US):

I. Accompanying this transmittal letter are certain items which are required under 35 U.S.C. 371 in order that United States National processing of the above identified International application may commence:

(X) at the expiration of the applicable time limit under PCT Articles 22 and 39(1) according to the provisions of 35 U.S.C. 371(b).

( ) as soon as possible upon receipt of this express request under 35 U.S.C. 371(f).

1. The U.S. National fee [35 U.S.C. 371(c)(1)]

a. ( ) was previously transmitted by applicant on (date)____.

b. ( ) is submitted herewith as follows:

FOR	NO. FILED	NO. EXTRA	SMALL ENTITY RATE	FEE	or	OTHER THAN SMALL ENTITY RATE	FEE
Basic Fee	(USPTO NOT ISA OR IPEA)		////	$485	or	/////	**$970**
Total Claims	- 20 =	--	x 9 =		or	x18 =	
Ind. Claims	**2 - 3**	--	x39 =		or	x78 =	
(**X**) Multiple Dependent Claim Presented			+130 =		or	+260 =	**260**
	TOTAL NATIONAL FEE		$____		or		$1230

i. ( ) A check in the amount of $_____ is enclosed.

ii. (**X**) Please charge the filing fee, multiple dependent claim fee (if applicable), excess independent claims fee (if applicable), and excess total claims fee (if applicable) to **Deposit Account No. 23-1703**.

iii. (X) The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 23-1703. A duplicate copy of this sheet is enclosed.

(iv) ( ) The filing fee is not enclosed.

2. A copy of the International application as filed [35 U.S.C. 371(c)(2)]:

a. (**X**) is transmitted herewith.

b. ( ) is not required as the application was filed with the United States Receiving Office.

c. ( ) has been transmitted

i. ( ) by the International Bureau. Date of mailing of the application (from form PCT/IB/308): _____ A copy of form PCT/IB/308 is enclosed.

ii. ( ) by applicant on (date) _____.

3. A translation of the International application into the English language [35 U.S.C. 371(c)(2)]:

a. ( ) is transmitted herewith.

b. (**X**) is not required as the application was filed in English.

c. ( ) was previously transmitted by applicant on (date)_____.

4. Amendments to the claims of the International application under PCT Article 19 [35 U.S.C. 371(c)(3)]:

a. ( ) are transmitted herewith.

b. ( ) have been transmitted

i. ( ) by the International Bureau. Date of mailing of the amendments (from form PCT/IB/308): _____.

ii. ( ) by applicant on (date) _____.

c. (**X**) have not been transmitted as

i. ( ) no notification has been received that the International Searching Authority has received the Search Copy.

ii. ( ) the Search Copy was received by the International Searching Authority but the Search Report has not yet issued. Date of receipt of Search Copy (from form PCT/ISA/202): _____.

iii. ( ) applicant chose not to make amendments under PCT Article 19. Date of mailing of Search Report (from form PCT/ISA/210):

iv. (X) the time limit for the submission of amendments has not yet expired. The amendments or a statement that amendments have not been made will be transmitted before the expiration of the time limit under PCT Rule 46.1.

5. A Translation of the amendments to the claims under PCT Article 19 [35 U.S.C. 371(c)(3)]:

a. ( ) is transmitted herewith.

b. ( ) is not required as the amendments were made in the English language.

c. (X) has not been transmitted for reasons indicated at point I.4.b. or c. above.

6. A declaration for patent application of the inventor [35 U.S.C. 371(c)(4)] complying with 35 U.S.C. 115:

a. ( ) was previously submitted by applicant on (date) _____

b. **(X)** is submitted herewith; and such oath or declaration

i. **(X)** is attached to the application.

ii. **(X)** identifies the application and any amendments under PCT Article 19 which were transmitted as stated in points 1.2.b. or c. and 1.4. and states that they were reviewed by the inventor as required by 37 CFR 1.70.

c. ( ) will be submitted subsequently.

II. Concerning other documents:

1. An International Search Report or Declaration under PCT Article 17(2)(a):

a. ( ) has been transmitted by the International Bureau. Date of mailing (from form PCT/IB/308): _____ A copy of form PCT/IB/308 is enclosed

b. ( ) is not required as the application was searched by the United States International Searching Authority.

c. ( ) A copy of the International Search Report is transmitted herewith.

d. ( ) has been submitted by applicant on (date) _____.

2. A Statement of prior art under 37 CFR 1.97 and 1.98:

a. ( ) is transmitted herewith including copies of the references cited on the attached form PTO-1449. Also included is a copy of the International-Type Search Report issued in the Swedish priority document.

b. ( ) will be transmitted within THREE MONTHS of the date of submission of requirements under 35 U.S.C. 371(c).

c. ( ) was previously submitted by applicant on _____, in application serial no. _____.

3. (**X**) An Assignment is transmitted herewith for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.

a. (**X**) Please charge the $40 assignment recordation fee to Deposit Account No. 23-1703.

b. ( ) A check in the amount of $___ is enclosed.

4. **Other document(s) or information included:**

- Copy of PCT/RO/101 - The PCT Request Form;
- Four sheets of drawings; and
- a return Postcard.

Respectfully submitted,

Richard J. Steiner

July 6, 1999
DATE

Richard J. Steiner
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Title: **NEW ASSAY**

Reference: R 1944

Inventors: SUNITA DESOUSA

PRAHLAD DWARAKANATH

NEW ASSAY

The present invention relates to a new assay for detecting peptidoglycan synthesis.

Peptidoglycan is a major component of the bacterial cell wall that gives the wall its shape and strength. It is unique to bacteria and found in all bacteria, both gram-positive and gram-negative. Peptidoglycan is a polymer of glycan strands that are cross-linked through short peptide bridges. It consists of alternating β1-4 linked residues of *N*-acetyl glucosamine (Glc*N*Ac) and *N*-acetyl muramic acid (Mur*N*Ac). A pentapeptide chain is attached to Mur*N*Ac (Mur*N*Ac-pentapeptide) and cross-linking occurs between these peptide chains.

Biosynthesis of peptidoglycan can be divided into three stages: firstly, synthesis of the precursors in the cytoplasm, secondly, transfer of the precursors to a lipid carrier molecule and, thirdly, insertion of the precursors into the cell wall and coupling to existing peptidoglycan.

The precursors synthesised in the cytoplasm are the sugar nucleotides: UDP-*N*-acetyl-glucosamine (UDP-Glc*N*Ac) and UDP-*N*-acetylmuramylpentapeptide (UDP-Mur*N*Ac-pentapeptide).

The second stage, which occurs in the cytoplasmic membrane, is catalysed by two enzymes and involves synthesis of a disaccharide unit on a lipid carrier, undecaprenyl phosphate. The lipid carrier is also involved in the synthesis of other components of the bacterial cell wall.

The first enzyme catalyses the transfer of phosphoryl-*N*-acetyl muramyl pentapeptide from UDP-Mur*N*Ac-pentapeptide to undecaprenol phosphate with the simultaneous release of UMP. This enzyme is called phospho-*N*-acetylmuramyl-pentapeptide translocase (hereafter referred to as "the translocase") and is the product of the gene mraY in

Escherichia coli. The product, undecaprenol-pyrophosphate-N-acetylmuramylpentapeptide (Lipid-P-P-MurNAc-pentapeptide) or Lipid I or Lipid linked precursor I is the substrate for the second enzyme.

N-acetylglucosaminyl transferase, transfers N-acetylglucosamine from UDP-GlcNAc (with simultaneous release of UDP) to form undecaprenol-pyrophosphoryl-*N*-acetylmuramylpentapeptide-*N*-acetylglucosamine or Lipid II or Lipid linked precursor II. This enzyme is also called UDP-*N*-acetylglucosamine: *N*-acetylmuramyl(pentapeptide)-P-P-undecaprenol-*N*-acetylglucosamine transferase (hereafter referred to as "the transferase"). The enzyme is the product of the gene murG in *Escherichia coli.*

The translocase and the transferase enzymes are essential for bacterial viability (see respectively D.S. Boyle and W.D. Donachie, J. Bacteriol. (1998), **180**, 6429-6432 and D. Mengin-Lecreulx, L. Texier, M. Rousseaue and J. Van Heijernoot, J. Bacteriol. (1991), **173**, 4625-4636).

In the third stage, at the exterior of the cytoplasmic membrane, polymerisation of the glycan occurs. The disaccharide-pentapeptide unit is transferred from the lipid carrier to an existing disaccharide unit or polymer by a peptidoglycan transglycosylase (also referred to as a peptidoglycan polymerase) (hereafter referred to as "the transglycosylase"). The joining of the peptide bridge is catalyzed by peptidoglycan transpeptidase (hereafter referred to as "the transpeptidase"). Both enzyme activities which are essential reside in the same molecule, the penicillin binding proteins (or PBPs), as in PBP 1a or 1b in *Escherichia coli.* These are the products of the ponA and ponB genes respectively, in *Escherichia coli.*

On transfer of the disaccharide-pentapeptide unit from the lipid precursor to an existing peptidoglycan chain the lipid is released as a molecule of undecaprenol pyrophosphate. This has to be cleaved by a bacitracin-sensitive undecaprenyl pyrophosphorylase, also called undecaprenol pyrophosphorylase or C55-isoprenyl

pyrophosphorylase (hereafter referred to as the "lipid pyrophosphorylase") to generate undecaprenol phosphate which can then re-enter the cycle at the second stage. Since inhibition of this enzyme will inhibit recycling of the lipid precursor it could also inhibit formation of peptidoglycan.

The transglycosylase is usually assayed by radiolabelling one of the sugar molecules and monitoring its incorporation into peptidoglycan. It is a difficult enzyme to assay because the lipid carrier molecule with bound disaccharide is neither simple to make nor water-soluble and, furthermore, the reaction only occurs on a solid phase (e.g. on Whatman 3 mm paper) and so the reaction conditions are difficult to control.

The transglycosylase activity may alternatively be assayed indirectly in a solution phase assay which, whilst being easier to control, requires the use of three of the other key enzymes involved in peptidoglycan synthesis, the translocase (e.g. the mraY gene product), the transferase (e.g. the murG gene product) and the lipid pyrophosphorylase.

In both types of assay, quantification of the products of enzymatic reaction is carried out using paper chromatography in which peptidoglycan stays at the origin and the reactants move away from the origin.

It would be desirable to develop an assay for detecting peptidoglycan synthesis which dispensed with the need for paper chromatography altogether. More particularly, it would be desirable to develop an assay for detecting peptidoglycan synthesis in which the reaction and quantification of the products of reaction could be performed entirely in the solution phase, for example, in a microtitre plate.

In accordance with the present invention, there is therefore provided an assay for detecting peptidoglycan synthesis, which comprises the steps of:

(1) incubating a reaction mixture comprising in aqueous medium a UDP-*N*-acetylmuramylpentapeptide (UDP-Mur*N*Ac-pentapeptide), radiolabelled UDP-*N*-acetyl

glucosamine (UDP-Glc*N*Ac), a source of divalent metal ions, a source of undecaprenyl phosphate, a source of peptidoglycan, a source of translocase enzyme (e.g. the *E.coli* mraY gene product), a source of transferase enzyme (e.g. the *E.coli* murG gene product), a source of transglycosylase enzyme, a source of transpeptidase enzyme (e.g. *E. coli* PBP 1a or PBP 1b) and a source of lipid pyrophosphorylase, under conditions suitable for peptidoglycan synthesis;

(2) adding a divalent metal ion chelator compound to the reaction mixture of step (1);

(3) adding lectin-coated beads impregnated with a fluorescer to the reaction mixture of step (2); and

(4) measuring light energy emitted by the fluorescer.

In the context of the present specification, it should be understood that the abbreviation "UDP" refers to uridine (5'-)diphosphate.

The assay according to the present invention is very conveniently carried out on 96-well microtitre plates, thereby enabling a fast, simple and reproducible way of measuring peptidoglycan synthesis.

In step (1), the UDP-Mur*N*Ac-pentapeptide used may be any of those usually present in naturally-occurring peptidoglycans and is conveniently purified from bacteria or made enzymatically with precursors from bacteria, e.g. by methods similar to that described by T. den Blaauwen, M. Aarsman and N. Nanninga, J. Bacteriol. (1990), **172**, 63-70).
A preferred UDP-Mur*N*Ac-pentapeptide to use is UDP-Mur*N*Ac-L-alanine-γ-D-glutamic acid-m-diaminopimelic acid-D-alanine-D-alanine from *Bacillus cereus*. The purified UDP-Mur*N*Ac-pentapeptide may also contain a certain amount of the tripeptide and tetrapeptide analogues and these may also participate effectively in the peptidoglycan synthesis reaction.

The concentration of UDP-Mur*N*Ac-pentapeptide used will typically be in the range from 50μM, preferably from 75μM, to 300μM, preferably 200μM, more preferably 100μM, per well of the microtitre plate.

As radiolabelled UDP-*N*-acetyl glucosamine, it is convenient to use tritiated UDP-*N*-acetyl glucosamine (UDP-[3H]Glc*N*Ac, commercially available from NEN-Dupont), preferably in a concentration of from 0.25 to 25μM per well of the microtitre plate, with radioactivity in the range from, e.g., 0.07 μCi to 2.00 μCi per well, preferably from 0.10 μCi to 1.00 μCi per well, and more preferably from 0.10 μCi to 0.5 μCi per well.

The divalent metal ions used are preferably magnesium ions. A suitable source of magnesium ions is magnesium chloride.

The membranes of *Escherichia coli* bacteria may conveniently be used and indeed are preferred as a source of undecaprenyl phosphate, peptidoglycan, translocase enzyme, transferase enzyme, transglycosylase enzyme, transpeptidase enzyme and lipid pyrophosphorylase enzyme. The quantity of membranes used will typically be in the range from 1 to 20μg, particularly from 4 to 6μg, protein per well of the microtitre plate. The membranes may be prepared by methods known in the art.

The aqueous medium used in step (1) is preferably a buffer solution, e.g. of Tris[hydroxymethyl]aminomethane hydrochloride ("Tris-HCl"), having a pH of about 7.5. Tris-HCl is commercially available from the Sigma-Aldrich Co. Ltd.

If the assay is intended to be used as a screen for identifying anti-bacterial compounds that are antagonists of the translocase, transferase, transglycosylase, transpeptidase or lipid pyrophosphorylase enzymes, the reaction mixture of step (1) may further comprise one or more test compounds in varying concentrations. Since the transglycosylase and transpeptidase enzymes are essential for bacterial growth and are located on the cell surface, these enzymes are regarded as especially good targets for the development of anti-

bacterial drugs as the drugs would not need to enter the bacterial organism through the cell wall and therefore the problems of cell wall permeability and also drug resistance brought about by changes in cell wall permeability are avoided.

The reaction mixture of step (1) is maintained at a temperature at or about 37 °C for a period of 0.5 to 4 hours, e.g. 1.5 hours, under conditions suitable for peptidoglycan synthesis to occur.

Peptidoglycan synthesis is terminated in step (2) by the addition of a suitable amount of a divalent metal ion chelator compound, e.g. ethylenediaminetetraacetic acid (EDTA) which is commercially available from the Sigma-Aldrich Co. Ltd. The concentration of the chelator compound will of course depend on the particular chelator compound used and should be sufficient to chelate all the divalent metal ions; in the case of EDTA the concentration will typically be about 15 mM per well of the microtitre plate.

In step (3), preferred lectin-coated beads impregnated with a fluorescer to use are those described in US Patent No. 4,568,649 and European Patent No. 154,734. The beads (known as "Scintillation Proximity Assay" (or SPA) beads) are commercially available from Amersham Inc. Most preferred are wheatgerm agglutinin-coated SPA beads which are capable of binding sugar molecules, specifically N-acetyl glucosamine. Thus, through the binding of N-acetyl glucosamine to the SPA beads, radiolabelled peptidoglycan formed in step (1) is brought into close proximity with the fluorescer which becomes activated by the radiation energy, resulting in the emission of light energy which is subsequently measured in step (4).

The beads which are conveniently added in the form of an aqueous suspension are contacted with the reaction mixture of step (2) for a period of 3 hours or more (e.g. overnight) before the plate is "counted" in step (4), e.g., in a "Microbeta Tilux" counter.

Apart from screening for anti-bacterial compounds as mentioned above, the assay according to the invention may, since it is sensitive to β-lactam antibiotics, be used alternatively to screen for novel β-lactams and also to measure the concentration of β-lactam antibiotics or to measure the activity of β-lactamases, enzymes that degrade β-lactams. In this way, the assay can be used as a diagnostic to detect disease-causing bacteria that are resistant to β-lactams because of the production of β-lactamases. Further, the assay may be used to identify inhibitors of β-lactamases, a key area of drug development.

The present invention will be further illustrated with reference to the following Example.

Example 1

(i) The wells of a microtitre plate were individually filled with a total volume of 25 µl of a reaction mixture comprising an aqueous buffer solution of 100 mM Tris[hydroxymethyl]aminomethane hydrochloride ("Tris-HCl") and 10 mM magnesium chloride (pH 7.5), 75 µM UDP-Mur*N*Ac-L-alanine-γ-D-glutamic acid-m-diaminopimelic acid-D-alanine-D-alanine, 2.5 µM tritiated UDP-*N*-acetyl glucosamine (0.5 µCi per well), 4 µg of *Escherichia coli* AMA1004 cell membranes and a solution of test compound (e.g. Tunicamycin, Vancomycin, Moenomycin, Penicillin G, Ampicillin, Cephaloridine and Bacitracin) of varying concentration in 4% dimethylsulphoxide. Tunicamycin is a known antagonist of the translocase enzyme, Vancomycin and Moenomycin are known antagonists of the transglycosylase enzyme, Penicillin G, Ampicillin and Cephaloridine are known antagonists of the transpeptidase enzyme and Bacitracin is a known antagonist of the lipid pyrophosphorylase.

Four wells of the microtitre plate were used as controls: two wells contained no UDP-*N*-acetylmuramylpentapeptide (0% reaction controls) and a further two wells contained no test compound (100% reaction controls).

The E. coli membranes were prepared in the following manner. Four to five colonies of the bacteria from an LB (Luria Bertani medium) agar plate were inoculated into 5 ml LB-broth and grown during the day (for 6-8 hours) at 37°C. In the evening 0.5 ml of this culture was used to inoculate 500 ml of LB-broth in a 2 l flask. The flask was incubated on a shaker at 30°C overnight; typically an A600 of 2.0-2.5 was reached. Early the next morning this culture was used to inoculate 6 l of LB-broth (using 500 ml of LB-broth per 2 l flask) such that the starting A600 was 0.4-0.6. The culture was grown for 2 hours at 37°C with vigorous shaking/aeration; the A600 reached was between 1.4 and 2.0. At this point the bacteria were cooled on ice and pelleted by centrifugation at 5,000 x g for 15 minutes. The cell pellet was washed with 500 ml of Buffer A (50 mM Tris-HCl, pH 7.5 / 0.1 mM $MgCl_2$) and resuspended in a minimal volume (< 20ml) of Buffer A. The cells were lysed using the French Pressure cell. The cell lysate was spun at 3,500 x g for 45 minutes. The supernatant was collected, diluted to 100 ml with Buffer A and ultra-centrifuged at 150,000 x g for 45 minutes. The pellet from this spin was washed by resuspending it in 100 ml of Buffer A and re-centrifuging at 150,000 x g for 30 minutes. This pellet was gently resuspended in a minimal volume (5-10 ml for 6 l culture) of Buffer A and frozen and stored in aliquots at -70°C. This is termed the membrane preparation and was used in the assay as a source of the peptidoglycan, five enzymes and undecaprenyl phosphate.

The microtitre plate was incubated at 37 °C for 1.5 hours and thereafter 5 µl of ethylenediaminetetraacetic acid (EDTA) was added to give a final EDTA concentration of 15 mM.

(ii) After addition of the EDTA, 170 µl of an aqueous suspension of wheatgerm agglutinin-coated scintillation proximity assay beads comprising 500 µg beads in a solution of Tris-HCl, pH 7.4, and t-octylphenoxypolyethoxyethanol ("Triton X-100", commercially sold by the Sigma-Aldrich Co. Ltd.) was added to each well such that the final concentration of Tris-HCl was 100 mM and that of Triton X-100 was 0.05%.

The plate was left for 3 hours at room temperature before being counted in the "Microbeta Trilux" counter.

Figure 1 is a graph showing the counts per minute (cpm) versus time based on the readings taken from the 100% controls.

Figure 2 is a graph showing the percentage inhibition of translocase (and thus peptidoglycan synthesis) versus Tunicamycin concentration.

Figure 3 is a graph showing the percentage inhibition of transglycosylase (and thus peptidoglycan synthesis) versus Vancomycin concentration.

Figure 4 is a graph showing the percentage inhibition of transglycosylase (and thus peptidoglycan synthesis) versus Moenomycin concentration.

Figure 5 is a graph showing the percentage inhibition of transpeptidase (and thus peptidoglycan synthesis) versus Penicillin G concentration.

Figure 6 is a graph showing the percentage inhibition of transpeptidase (and thus peptidoglycan synthesis) versus Ampicillin concentration.

Figure 7 is a graph showing the percentage inhibition of transpeptidase (and thus peptidoglycan synthesis) versus Cephaloridine concentration.

Figure 8 is a graph showing the percentage inhibition of lipid pyrophosphorylase (and thus peptidoglycan synthesis) versus Bacitracin concentration.

CLAIMS

1. A Scintillation Proximity Assay (SPA) for the detection of peptidoglycan synthesis.

2. An assay for detecting peptidoglycan synthesis, which comprises the steps of:
(1) incubating a reaction mixture comprising in aqueous medium a UDP-*N*-acetylmuramylpentapeptide, radiolabelled UDP-*N*-acetyl glucosamine, a source of divalent metal ions, a source of undecaprenyl phosphate, a source of peptidoglycan, a source of translocase enzyme, a source of transferase enzyme, a source of transglycosylase enzyme, a source of transpeptidase enzyme and a source of lipid pyrophosphorylase enzyme, under conditions suitable for peptidoglycan synthesis;
(2) adding a divalent metal ion chelator compound to the reaction mixture of step (1);
(3) adding lectin-coated beads impregnated with a fluorescer to the reaction mixture of step (2); and
(4) measuring light energy emitted by the fluorescer.

3. An assay according to claim 2, wherein the UDP-*N*-acetylmuramylpentapeptide is UDP-Mur*N*Ac-L-alanine-γ-D-glutamic acid-m-diaminopimelic acid-D-alanine-D-alanine.

4. An assay according to claim 2 or claim 3, wherein bacterial cell membranes represent a source of one or more of undecaprenyl phosphate, peptidoglycan, translocase enzyme, transferase enzyme, transglycosylase enzyme, transpeptidase enzyme and lipid pyrophosphorylase enzyme.

5. An assay according to claim 4, wherein the bacterial cell membranes are from *Escherichia coli.*

6. An assay according to any one of claims 2 to 6, wherein the reaction mixture of step (1) further comprises a test compound.

7. An assay according to claim 6, wherein the test compound is an antagonist of one of the enzymes.

8. An assay according to any one of claims 2 to 7, wherein ethylenediaminetetraacetic acid is used as the divalent metal ion chelator compound in step (2).

9. An assay according to any one of claims 2 to 8, wherein the lectin-coated beads comprise wheatgerm agglutinin.

ABSTRACT

NEW ASSAY

The invention provides a scintillation proximity assay for detecting peptidoglycan synthesis. The assay is especially suitable for high throughput screening of compounds affecting peptidoglycan synthesis.

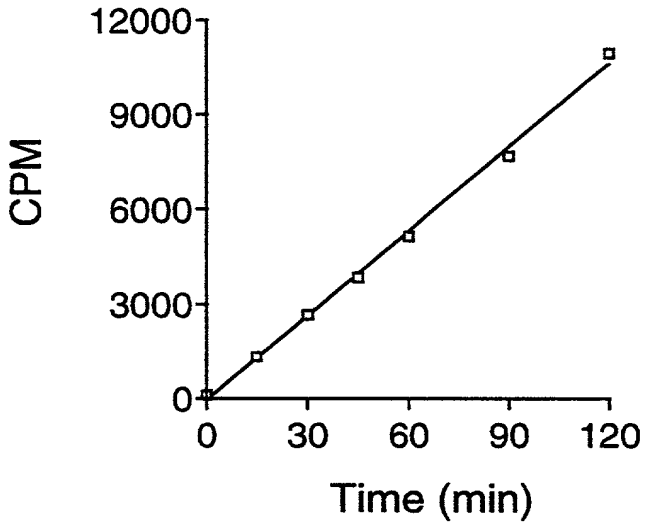

Figure 1

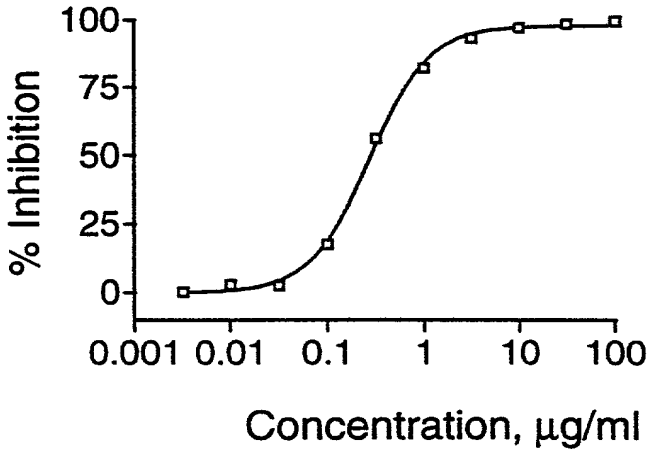

Figure 2

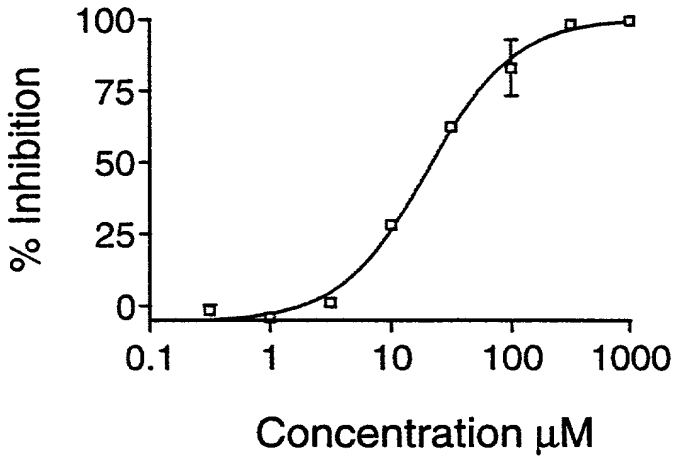

Figure 3

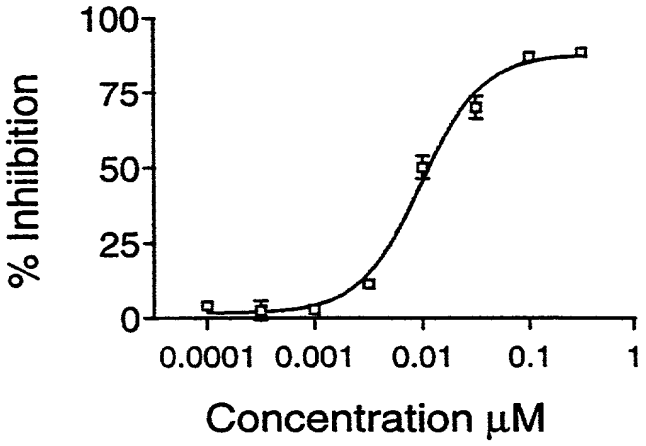

Figure 4

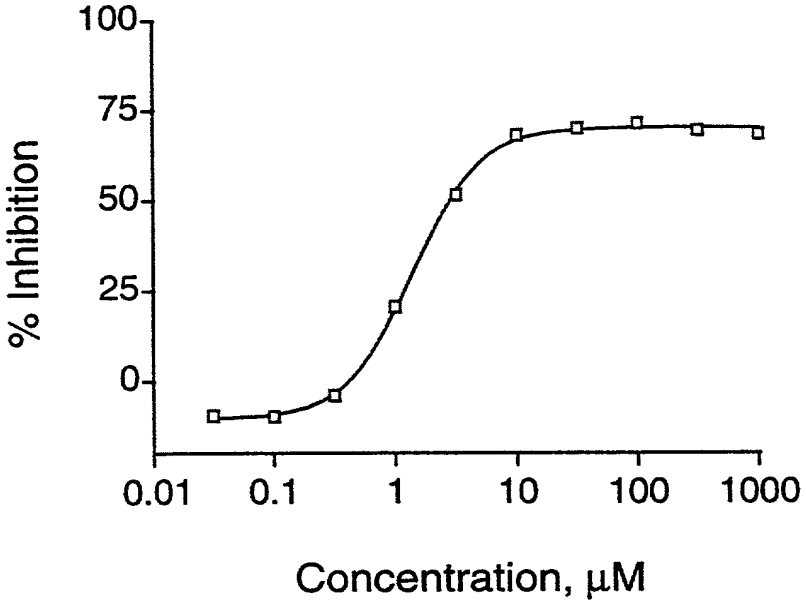

Figure 5

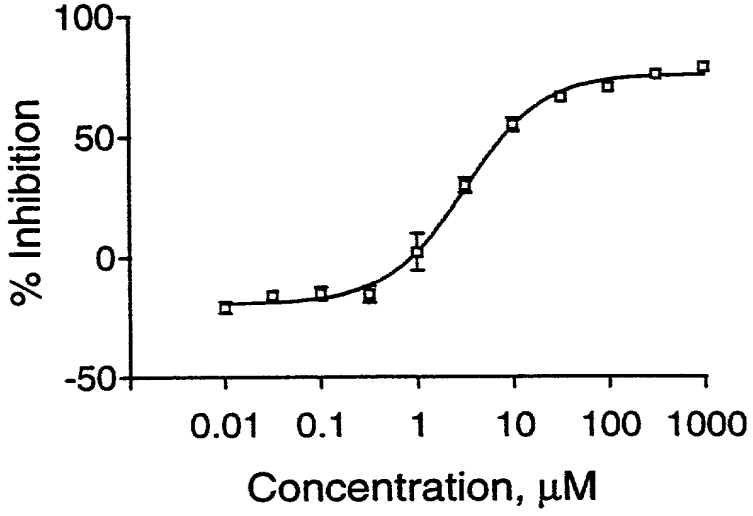

Figure 6

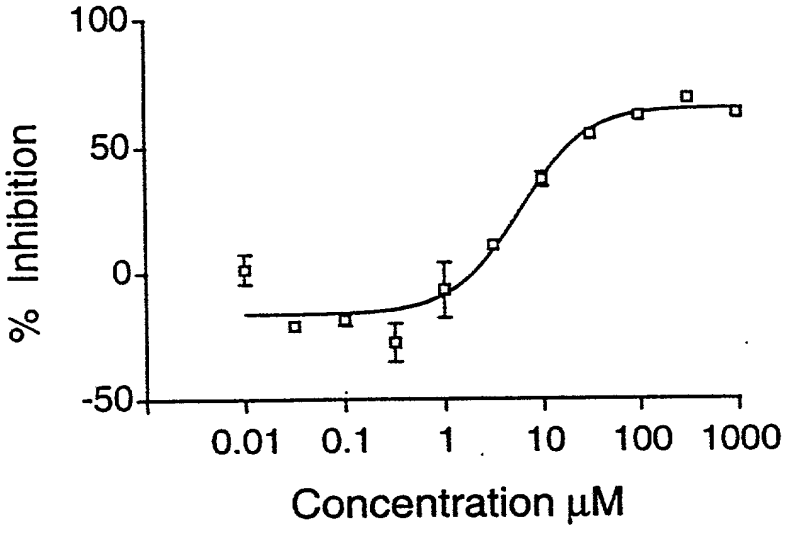

Figure 7

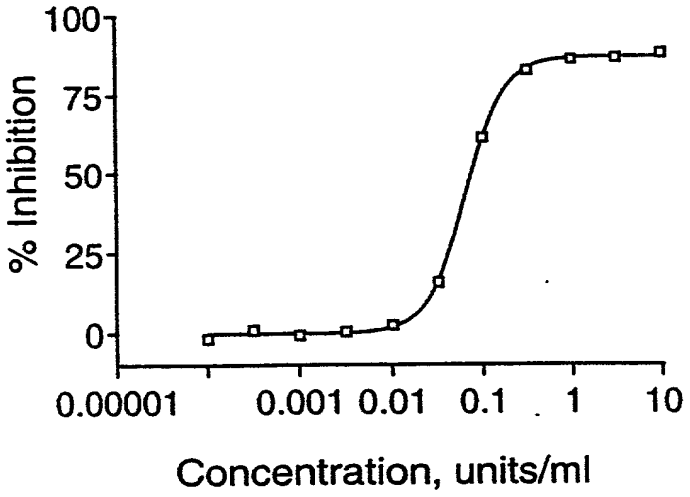

Figure 8

Docket Number (Optional) R 1944-1P US

DECLARATION FOR PATENT APPLICATION

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled NEW ASSAY the specification of which is attached hereto unless the following box is checked:

☒ was filed on 04 May 1999 as United States Application Number or PCT International Application Number SE99/00749 and was amended on (if applicable).

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR § 1.56

I hereby claim foreign priority benefits under 35 U.S.C. § 119(a)-(d) or § 365(b) of any foreign application(s) for patent or inventor's certificate, or § 365(a) of any PCT International application which designated at least one country other than the United States, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or PCT international application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application(s)

			Priority Not Claimed
1019/MAS/98 (Number)	India (Country)	15 May 1998 (Day/Month/Year Filed)	☐
9802210-6 (Number)	Sweden (Country	22 June 1998 (Day(Month/Year Filed)	☐

I hereby claim the benefit under 35 U.S.C. § 119(e) of any United States provisional application(s) listed below.

(Application Number)	(Filing Date)
(Application Number	(Filing Date)

I hereby claim the benefit under 35 U.S.C. § 120 of any United States application(s), or § 365(c) of any PCT International application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of 35 U.S.C. § 112.

I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR § 1.56 which became available between the filing date of the prior application and the national or PCT International filing date of this application.

(Application Number)	(Filing Date)	(Status -- patented, pending, abandoned)
(Application Number)	(Filing Date)	(Status -- patented, pending, abandoned)

I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith:

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believe to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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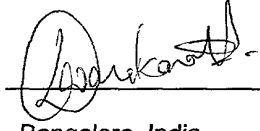
Second inventor's signature  Date 21 June 1999

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